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Antimicrobial activity of flavonoids and steroids isolated from two *Chromolaena* species

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The crude extracts (dichloromethanic and ethanolic) and some compounds (8 flavonoids and 5 steroids) isolated from Chromolaena squalida (leaves and stems) and Chromolaena hirsuta (leaves and flowers) have been evaluated against 22 strains of microorganisms including bacteria (Gram-positive and Gram-negative) and yeasts. All crude extracts, flavonoids and steroids evaluated have been shown actives, mainly against Gram-positive bacteria.

Unitermos
- *Chromolaena*
- Asteraceae
- Flavonoids
- Steroids
- Antimicrobial activity

INTRODUCTION

Flavonoids are phenolic substances widely distributed in all vascular plants. They are a group of about 4000 naturally compounds known, and have been shown to have contribute to human health through our daily diet. They are ubiquitous in plant foods and drinks such as fruits, vegetables, tea, wine, coffee and beer (Giulia *et al*., 1999).

In a review, discussed by Harborne and Willians (2000), many studies have suggested that flavonoids exhibit antioxidant, anti-inflamatory, antimicrobial, vascular activities and others medicinal properties. Many reports on the antimicrobial activity of flavonoids are available (Baez *et al*., 1999; Xu, Lee, 2001; Ogundipe *et al*., 2001). Related studies of antimicrobial activity indicate that crude extracts containing flavonoids, triterpenes and steroids have showed significative activity against various strains of *Staphylococcus aureus, Streptococcus faecalis* and *Escherichia coli* (Chattopadhay *et al*., 2001).

Antibacterial effect against eight strains of Gram-positive and Gram-negative bacteria (Minimum Inhibitory Concentration (MIC) in the range of 64 to 250 μg/mL) was showed for crude extract of *Castanea sativa*. The analyse by TLC and HPLC of the active fraction showed the presence of flavonoids rutin, hesperidin, quercetin, apigenin, morin, naringin, galangin and kaempferol. Standards of these flavonoids were assayed against the same bacterial strains, and the highest activity was shown by quercetin, rutin and apigenin (Basile *et al*., 2000).

Antimicrobial screening of 13 phenolic substances was carried out by diffusion methods against *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Staphylococcus nervous* and *Staphylococcus epidermidis*. The flavonoids flavone, quercetin and naringenin inhibited the growth of these organisms (Rauha *et al*., 2000).

The ocurrence of flavonoids on crude extracts of *C. squalida* and *C. hirsuta* (Asteraceae-Eupatorieae) is of the great interest to discover new plant derived-compounds. It lead us to evaluate the antimicrobial activity of these extracts and some isolated compounds.

MATERIAL AND METHODS

General experimental procedure

The IR spectra were obtained on KBr pellets in a Perkin Elmer model 1420 spectrophotometer. ^{1}H NMR (300 MHz) and the ^{13}C NMR (75 MHz) spectra were recorded on a Brucker Avance DPX 300; and the ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Brucker-Avance DPX 400, in $CDCl_3$ and DMSO-d_6 with TMS as internal standard. The UV spectra were obtained in Hitachi U-3501 spectrophotometer. TLC was carried out on Si gel PF-254 (Merck), CC on Si gel 60 (0.063-0.200), (Merck) and CC on Sephadex LH 20 (Sigma, 25-100 µ).

Plant material of *Chromolaena squalida*

Aerial parts (leaves and stems) of *C. squalida* (ex *E. squalidum*) was collected by Prof. Dr. N. P. Lopes in Furnas, a town in the state of Minas Gerais - Brazil, in April 1998, and identified by Prof. Dr. E. E. Schilling (University of Tennessee – Knoxville, TN, USA) and Prof. Dr. H. Robinson (Department of Botany, Smithsonian Institute, Washington D.C., USA). The voucher specimen (NPL 126) was deposited in the Herbarium of the Department of Biology, FFCLRP/USP, Brasil, SPFR 04414.

Extraction and preparation of test solutions

Test solutions were prepared in DMSO/sterile water (5:95) at 1000 µg/mL for the crude extracts from *C. squalida* (leaves and stems) and *C. hirsuta* (leaves and flowers) and at 500 µg/mL for each isolated compounds (flavonoids and mixture of steroids).

Isolation of compounds from *Chromolaena* species

Dried and pulverized leaves (73 g) and stems (64 g) of *C. squalida* were extracted at room temperature with CH_2Cl_2 and then EtOH, separately, to give the respective crude extracts.

The CH_2Cl_2 crude extract of leaves **Csd1** (3.91 g) and stem **Csd2** (0.75 g) were chromatographed over Silica gel 60 (CC), eluting with hexane and gradually increasing the polarity with EtOAc and then MeOH.

From the **Csd1** crude extract was extracted the flavonoid **1** (0.007 g). The crude extract **Csd2** furnished the mixture (0.012 g) of steroids stigmasterol **9**, β-sitosterol **10**, campesterol **11**, espinasterol **12**, Δ^7-stigmastenol **13**.

The EtOH crude extract of leaves **Cse1** (2.08 g) was chromatographed on Sephadex LH-20, eluting with MeOH. All collected fractions were monitored by TLC, and the reunited fractions were purified by chromatographic process. This extract furnished the flavonoid **2** (0.012 g).

Dichloromethanic and ethanolic crude extracts (leaves and flowers) of *C. hirsuta* were previously studied (Taleb-Contini, 2002; Taleb-Contini, Oliveira, 2000). It resulted in isolation of the flavonoids **3-8** which were, in this study, evaluated for antimicrobial activity.

The structures of the flavonoids are presented on Figure 1.

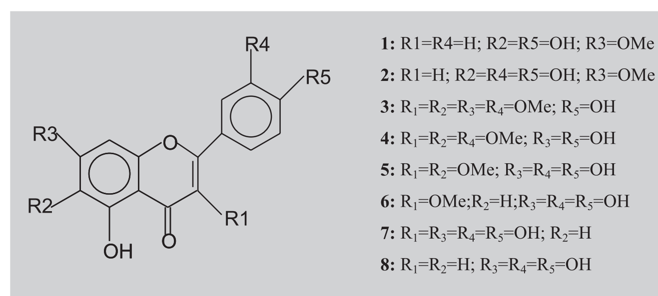

FIGURE 1 - Flavonoids from *Chromolaena* species evaluated for antimicrobial activity.

Microorganisms strains

Twenty two strains of bacteria (Gram-positive and Gram-negative) and yeasts were used in the antimicrobial assays. The following microorganisms were used: *Escherichia coli* - ATCC 10538; *E. coli* - 26.1 (field strain); *Pseudomonas aeruginosa* - ATCC 27853; *P. aeruginosa* - Pn (field strain); *Micrococcus luteus* - ATCC 9341; *Staphylococcus aureus* - ATCC 25923, 6538 and 29213; *S. aureus* - 7+ penicillinase producer; *S. aureus* - 8-penicillinase non-producer; *Staphylococcus epidermidis* - 6ep (field strains); *Candida albicans* - ATCC 1023; *Candida albicans*-cas and *Candida tropicalis* - ct (field strains), cultivated for 24 hours at 37 °C in Mueller Hinton broth (Difco)-MHb; *Enterococcus faecalis* - ATCC 10541; *Streptococcus mutans* - ATCC 25175; *S. mutans* (strains Fab3; 87.1; 203.1; 211.1; 213.1) and *Streptococcus sobrinus* - 87.3 (field strains) incubated for 24 hours at 37 °C in Brain Heart Infusion (Difco) - BHI. The standart strains and field strains (oral cavity) were collected from Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto (SP), Brasil.

Determination of antimicrobial activity

The inoculum size of each test strain was standardized according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993). The test bacterial and yeasts strains were inoculated into Mueller Hinton broth (Difco) - MHb agar plates (*Escherichia*, *Pseudomonas*, *Micrococcus*, *Staphylococcus* and *Candida* strains) and Brain Heart Infusion (Difco)-BHI plates (*Enterococcus* and *Streptococcus* strains), containing an inoculum size of 10^6 cfu/mL (0.5 McFarland scale).

Antimicrobial activity was performed by the well diffusion method (well technique in double layer) (Cole, 1994; Grove, Randall, 1955).

A volume the 20 µL of each test-drug solution were applied into 5.0 mm diameter wells. After holding the plates at room temperature for 2 hours to allow diffusion of test-drug into the agar, they were incubated at 37 °C for 24 hours and the inhibition zone, corresponding to the halo (H) formed from well edge to the beginning of the region of microbial growth was measured in millimeters (mm). The MIC was determined in µg/mL for each isolated compound with concentration between 25 and 500 µg/mL (Salvador *et al*., 2002; Okeke *et al*., 2001; Okunji *et al*., 1990). In these tests, bacitracine (0.2 UI/mL), gentamicine discs (10 µg) and ketoconazole (100 µg/mL) were used as experimental positive controls for microorganism strains and DMSO/sterile water (5:95) as negative control for which no inhibitory effect could be observed. The bioassays were performed in duplicate for each strain of microorganism evaluated.

RESULTS AND DISCUSSION

The identities of steroids stigmasterol, β-sitosterol, campesterol, espinasterol and Δ^7-stigmasterol were confirmed by GC analysis, using authentic samples.

The bioactive flavonoids **1** and **2** were caracterized by comparing physical and spectroscopic properties (^{1}H,

TABLE I - Antimicrobial activity of crude extracts of *C. squalida* (leaves and stem) and *C. hirsuta* (leaves and flowers)

Microorganisms	Csd1	Cse1	Csd2	Cse2	Chd1	Che1	Chd3	Che3	B	G	K
	H	H	H	H	H	H	H	H	H	H	H
M. luteus (ATCC 9341)[b]	-	-	-	-	-	-	-	-	25	28	-
S. aureus (ATCC 6538)[b]	7	-	8	-	6	9	13	6	23	26	-
S. aureus (ATCC 25923)[b]	6	-	7	-	6	7	10	-	29	28	-
S. aureus (ATCC 29213)[b]	8	-	-	-	6	8	9	-	29	28	-
S. aureus (7+)[c]	8	7	8	-	6	7	11	7	25	28	-
S. aureus (8-)[c]	8	-	7	-	7	8	13	6	25	26	-
S. epidermidis (6ep)[c]	6	-	6	-	7	7	12	-	31	27	-
E. faecalis (ATCC 10541)[b]	-	-	-	-	-	-	8	-	28	22	-
S. mutans (ATCC 25175)[b]	-	-	-	-	6	7	-	-	24	23	-
S. mutans (Fab3)[b]	-	-	6	-	6	8	-	-	24	24	-
S. mutans (87.1)[c]	6	6	-	-	6	7	7	6	24	20	-
S. mutans (203.1)[c]	-	-	-	-	7	7	6	6	22	21	-
S. mutans (211.1)[c]	-	-	-	-	-	-	-	6	22	20	-
S. mutans (213.1)[c]	-	-	-	-	6	-	-	-	22	22	-
S. sobrinus (87.3)[c]	-	-	6	6	-	7	-	-	23	24	-
E. coli (ATCC 10538)[b]	-	-	-	-	-	-	-	-	30	30	-
E. coli (26.1)[c]	-	-	-	-	-	-	-	-	32	30	-
P. aeruginosa (ATCC 27853)[b]	-	-	-	-	-	-	-		22	25	-
P. aeruginosa (Pn)[c]	-	-	-	-	-	-	-	-	22	24	-
C. albicans (ATCC 1023)[b]	-	-	-	-	-	-	-	-	-	-	30
C. albicans (cas)[c]	-	-	-	-	-	-	-	-	-	-	30
C. tropicalis (ct)[c]	-	-	-	-	-	-	-	-	-	-	15

- = absence of inhibition of microbial growth at 1000 mg/mL; H = halo of inhibition (mm); Diluent = DMSO/sterile water (5:95); B = Bacitracine, 0.2 UI/mL; G = Gentamicine discs, 10 mg; K = Ketoconazole, 100 mg/mL; Csd1 = *C. squalida* dichloromethanic leaves; Csd2 = *C. squalida* dichloromethanic stem; Cse1 = *C. squalida* ethanolic leaves; Cse2 = *C. squalida* ethanolic stem; Chd1 = *C. hirsuta* dichloromethanic leaves; Chd3 = *C. hirsuta* dichloromethanic flowers; Che1= *C. hirsuta* ethanolic leaves; Che3 = *C. hirsuta* ethanolic flowers crude extracts; [b]=standard strains; [c] = field strains (oral cavity).

^{13}C NMR, IR and UV) spectra, with those reported in the literature, i.e. **1** (Silva *et al*., 1977), **2** (Saúl-Escarria *et al*., 1977). The spectral date of ^{1}HNMR showed the proton resonances commoly found in flavonoids and their substituents. Ultraviolet-visible absorption spectroscopy was useful to aid both identification of the flavonoid type and definition of the oxygenation pattern.

All the crude extracts (ethanolic and dichloromethanic) from leaves and stems of *C. squalida* and leaves and flowers from *C. hirsuta* showed antimicrobial activity, mainly against Gram-positive (*Staphylococcus* and *Streptococcus*) bacteria, at 1 000 mg/mL (Table I).

The evaluated compounds showed actives at least against two indicative strains used (Table II). The flavonoids **6**, **7** and **8** were the most bioactives. The mixture of steroids (500 mg/mL) showed to be active mainly against *Streptococcus mutans* and *S. sobrinus* strains, however with very limited activity. The flavonoids **1** and **2** were active against *S. aureus* (ATCC 6538 and ATCC 29213), *S. sobrinus* (87.3) and *Enterococcus faecalis* (ATCC-10541) strains.

From all test-drug only the flavonoid **7** showed activity against the Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria and yeast (*Candida albicans* and *C. tropicalis*).

The results reveal that the most bioactive flavonoids are those that have dyhydroxy groups at C3' and C4' positions, a hydroxy substituent at position C7, and the position C6 unsubstituted.

The structure-activity relantionships become very interesting when we compare the structures of flavonoids **6**, **7** and **8**. They all have a group hydroxy at positions C7, C3' and C4', but only the flavonoid **7** (that exhibited activity against Gram-negative bacteria and yeast) has a hydroxy group at C3 position. The flavonoids **1** and **2** that show the same substitution pattern of A ring and the C3 unsubstituted, were found to be active against the same bacterial strains, problaby acting by the same mechanism of action. However, it is necessary to make profound studies to better understand the mechanism of action of these evaluated substances.

CONCLUSION

Ethanolic and dichloromethanic crude extracts from leaves and stems of *C. squalida* and leaves and flowers from

TABLE II - Antimicrobial activity of compounds isolated from *C. squalida* and *C. hirsuta*

Microorganisms	Evaluated material MIC (µg/mL)								
	1	2	3	4	5	6	7	8	9-13
M. luteus (ATCC 9341)[b]	-	-	100	500	50	-	500	-	-
S. aureus (ATCC 6538)[b]	100	500	-	500	500	100	100	500	-
S. aureus (ATCC 25923)[b]	-	500	-	-	500	-	100	100	-
S. aureus (ATCC 29213)[b]	100	500	100	-	-	100	100	-	-
S. aureus (7+)[c]	-	-	500	-	-	500	100	500	500
S. aureus (8-)[c]	-	-	-	-	-	100	100	-	-
S. epidermidis (6ep)[c]	500	500	-	-	-	100	100	-	-
E. faecalis (ATCC 10541)[b]	500	500	-	-	-	500	100	-	-
S. mutans (ATCC 25175)[b]	-	-	-	-	-	-	500	500	500
S. mutans (Fab3)[b]	-	-	-	-	-	-	50	-	-
S. mutans (87.1)[c]	-	-	-	-	-	100	100	-	500
S. mutans (203.1)[c]	-	-	-	-	-	500	-	-	500
S. mutans (213.1)[c]	-	-	-	-	-	500	100	-	-
S. mutans (211.1)[c]	-	-	-	-	-	500	500	-	-
S. sobrinus (87.3)[c]	500	500	-	-	500	100	100	500	500
E. coli (ATCC 10538)[b]	-	-	-	-	-	-	500	500	-
E. coli (26.1)[c]	-	-	-	-	-	-	100	500	-
P. aeruginosa (ATCC 27853)[b]	-	-	-	-	-	-	100	-	-
P. aeruginosa (Pn)[c]	-	-	-	-	-	-	100	-	-
C. albicans (ATCC 1023)[b]	-	-	-	-	-	-	500	-	-
C. albicans (cas)[c]	-	-	-	-	-	-	500	-	-
C. tropicalis (ct)[c]	-	-	-	-	-	-	500	-	-

- = absence of inhibition of microbial growth until 500 mg/mL; MIC = Minimum Inhibitory Concentration (mg/mL); [b] = standard strains; [c] = field strains (oral cavity)

C. hirsuta showed antimicrobial activity, mainly against Gram-positive (*Staphylococcus* and *Streptococcus*) bacteria.

All the evaluated compounds showed to be bioactive, mainly the flavonoids **6**, **7** and **8**.

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RESUMO

Atividade antimicrobiana de flavonóides e esteróides isolados de duas espécies de *Chromolaena*

Os extratos diclorometânicos e etanólicos de folhas e caule de Chromolaena squalida e de folhas e flores de Chromolaena hirsuta foram avaliados quanto à atividade antimicrobiana. Oito flavonóides e cinco esteróides foram desafiados frente a 22 cepas indicadoras, incluindo bactérias (Gram-positivas e Gram-negativas) e leveduras. Todos os extratos brutos, flavonóides e esteróides ensaiados mostraram atividade antimicrobiana, principalmente frente a bactérias Gram-positivas.

UNITERMOS: Chromolaena. *Asteraceae. Flavonóides. Esteróides. Atividade antimicrobiana.*

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